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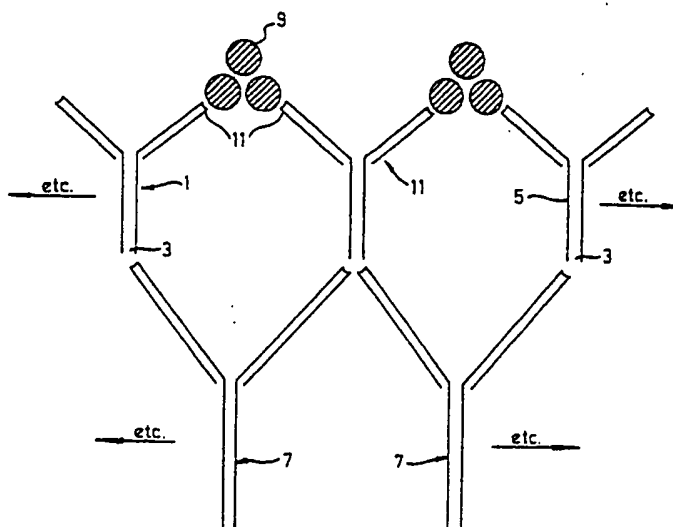
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(54) Title: MULTIVALENT ANTI-CYTOKINE IMMUNOGLOBULINS



(57) Abstract

A multivalent immunoglobulin which comprises at least three linked antigen binding domains each of said domains being specific for a complementary site on a cytokine, (e.g. TNF- α , TNF- β , an interleukin, an interferon, such as IFN- γ , or/and a colony stimulating factor) and characteristically interacts with the cytokine to neutralise its biological activity. The multivalent immunoglobulins have surprisingly increased neutralising activity and are useful for therapeutic or prophylactic treatment of conditions involving undesirably elevated levels of the cytokine. Preferably the multivalent immunoglobulin comprises from 4 to 20 antigen binding domains, which are especially of class IgG. The multivalent immunoglobulin may comprise recombinant immunoglobulin molecules and fragments thereof, and the antigen binding domains may be covalently or non-covalently linked.

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MULTIVALENT ANTI-CYTOKINE IMMUNOGLOBULINSFIELD OF THE INVENTION

5 The present invention relates to multivalent anti-cytokine immunoglobulins and to their manufacture and use in therapy. In particular, the multivalent immunoglobulins are suitable for use in therapies requiring the neutralisation of cytokines occurring in the body at deleteriously high levels.

10 The cytokines are a group of polypeptide mediators which transmit signals from one cell to another of the same or different type. As used herein the term cytokine encompasses lymphokines. For example, the anti-cytokine immunoglobulin may be an anti tumour necrosis factor- α (TNF- α) or anti tumour necrosis factor- β (TNF- β) immunoglobulin and may be employed in the reduction of
15 levels of TNF- α or TNF- β in the body.

BACKGROUND OF THE INVENTION

Antibodies, or immunoglobulins, are classified in five immunoglobulin classes, immunoglobulin G (IgG),
20 immunoglobulin M (IgM), immunoglobulin A (IgA), immunoglobulin D (IgD) and immunoglobulin E (IgE). Immunoglobulins of each of these classes comprise a basic four chain structure of two identical light polypeptide chains and two identical heavy polypeptide chains linked
25 together by disulphide bonds. This basic structure is hereinafter sometimes referred to as an immunoglobulin molecule. Each immunoglobulin class is further classified into subclasses. For example human IgG comprises subclasses IgG1, IgG2, IgG3 and IgG4. The
30 class and subclass of an immunoglobulin is determined by its heavy chain type.

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IgG is the major immunoglobulin in normal human serum and is a monomer of the four chain structure described above. IgM accounts for a smaller, but significant, proportion of the human immunoglobulin pool and is a
5 pentameric structure in which monomers of the four chain structure are linked by disulphide bridges between the heavy chains and by a polypeptide linker designated the J chain.

Of the other immunoglobulins, IgA is the most common and
10 may occur in either a monomeric or dimeric form. IgD and IgE occur in very small quantities in humans and are both exclusively monomeric.

The amino terminal domains of the heavy and light chains of the basic structure are characterised by sequence
15 variability and are designated V_H (variable heavy) domain and V_L (variable light) domain. The V_H and V_L domains together form antigen binding domains of the immunoglobulin. The basic four chain immunoglobulin structure thus has two antigen binding domains.
20 Consequently an IgM has a total of ten antigen binding domains. It will, therefore, be apparent that immunoglobulins occurring in nature have a variety of valencies for the antigens they bind, two in the case of IgG, IgD and IgE, two or four for IgA, and ten for IgM.

25 In a similar way, the antigens which an antibody recognises may have one, a few or many antigenic determinants or epitopes. The antigenic determinants of a multi determinant antigen may be the same as, or different from, one another.

30 For example, a small molecule or hapten may have a single antigenic determinant which is recognised by a

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complementary antigen binding region of an immunoglobulin molecule. By contrast a large protein molecule may have multiple, for example tens of, determinants on its surface.

5 It is already well known that the existence of antigens with multiple epitopes may lead to a bonus effect in which the binding of two antigen molecules by antibodies recognising different determinants on the antigen is always greater than the arithmetic sum of the links formed by the individual antibodies. The same considerations
10 are also known to apply to antibody binding to a polymeric antigen with the same repeating determinants. Thus the apparent avidity of the antibody - antigen interaction can be greater than the affinity of the individual antigenic determinant - antibody binding site interactions. As one
15 moves from a univalent Fab fragment possessing one V_H and one V_L domain to a divalent IgG to a pentameric IgM, the bonus effect of multivalency produces striking increases in the strength of antigen - antibody complex formation (See for example "Essential Immunology", I. Roitt 6th Edn
20 (1988) Blackwell Scientific Publications).

In the course of comparing various monoclonal antibodies to tumour necrosis factor- α (anti-TNF antibodies) with the same V_H domain for their ability to neutralise human recombinant TNF we observed that an IgM antibody was
25 significantly better than the best of the IgG antibodies tested. Furthermore, it was observed that a monoclonal IgG to recombinant TNF which had been stored for one year after manufacture and which had aggregated to higher molecular weight, including dimeric and trimeric, forms
30 displayed greater neutralisation activity than freshly manufactured monomeric antibody. Also, while the dimeric aggregate showed slightly better neutralisation than the

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monomer, the trimer gave much better neutralisation. These observations led us to cross link the available IgGs. The effect of the cross-linking was to produce a multivalent immunoglobulin and bring about a surprising increase in the neutralising activity of the antibodies.

DESCRIPTION OF ASPECTS OF THE INVENTION

Thus, according to one aspect of the present invention there is provided a multivalent immunoglobulin comprising at least three linked antigen binding domains each of said antigen binding domains being specific for a complementary site on a cytokine.

The combined interactions between the antigen binding domains of the multivalent immunoglobulin and the complementary cytokine sites are characteristically neutralising interactions. As used herein the expression "neutralising" or "neutralisation" means the inhibition of or reduction in a biological activity of the cytokine as measured in an in vitro or in vivo test.

Each of the at least three linked antigen binding domains are specific for the same cytokine. However, different antigen binding domains in the multivalent immunoglobulin may be specific for different epitopes of the same cytokine. For example, each antigen binding region is directed to a neutralising epitope of the cytokine which it recognises. However this need not be so, so long as the combined interactions of antigen binding domains and complementary sites are neutralising as mentioned above. In addition to the at least three linked antigen binding domains specific for the same cytokine, the multivalent immunoglobulin may include one or more other antigen binding domains specific for a further cytokine or other molecule.

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The cytokines for which the multivalent immunoglobulins of the invention are specific are typically soluble cytokines.

Examples of cytokines for which the antibody binding domains of the multivalent immunoglobulin may be specific include tumour necrosis factor- α (TNF- α), tumour necrosis factor- β (TNF- β or lymphotoxin), the interleukins (IL-1; IL-2, IL-3, IL-4, IL-5, IL-6 etc.), interferons (e.g. IFN- α , IFN- β or IFN- γ) and colony stimulating factors (e.g. G-CSF, GM-CSF, etc.).

10 The cytokine may be monomeric and have at least two epitopic sites recognised by one or more of the antigen binding domains of the multivalent immunoglobulin.

Alternatively, the antigen binding domains of the multivalent immunoglobulin may be specific for a
15 multimeric cytokine, such that the cytokine recognised may have at least two epitopic sites recognised by one or more of the antigen binding domains of the multivalent immunoglobulin. The cytokine may be multimeric as a result of its aggregation or polymerisation. For
20 example, TNF- α is known to exist in oligomeric as well as monomeric forms. Thus, TNF- α molecules associate with each other to form trimers and hence may behave as multivalent binding partners for the multivalent immunoglobulin. Interferon- γ and TNF- β may also exist in
25 oligomeric forms.

The size of the multivalent immunoglobulin is likely to be limited by solubility considerations. Thus, the number of linked antigen binding domains is preferably sufficiently small that the multivalent immunoglobulin
30 does not form an insoluble precipitate before it comes into contact with its complementary cytokine. The upper

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limit on the number of linked antigen binding domains may, perhaps be as many as 4000. However, the preferred number of linked antigen binding domains is likely to be much smaller than that, for example in the range from 4 to 20, preferably 4 to 10 antigen binding domains.

The antigen binding domains of the polymeric immunoglobulin may be derived from immunoglobulins of any class - IgG, IgM, IgA, IgD or IgE, and of any immunoglobulin subclass. The various antigen binding domains in a given polymeric immunoglobulin may be of the same or different immunoglobulin class or subclass. Preferably, they are all of class IgG.

Alternatively, all antigen binding domains in the multivalent immunoglobulin may be derived from IgM. In that case, the multivalent immunoglobulin differs from the native IgM molecule, for example in containing fewer than or greater than ten antigen binding domains, or in another way as will become apparent hereafter. Similarly, where the antigen binding domains are all derived from IgA the multivalent immunoglobulin differs from the native molecule. For example, the multivalent immunoglobulin may contain three or at least five binding domains or differ in some other way from native IgA.

The antigen binding domains of the multivalent immunoglobulin may be present as part of whole immunoglobulin molecules, for example of a whole IgG molecule. Alternatively, they may be part of immunoglobulin molecule fragments produced, for example, by enzymatic digestion of the intact molecule. Thus the antibody binding domains may be comprised by Fv, Fab, Fab' or F(ab')₂ fragments of IgG. Alternatively, the binding sites may be present on corresponding IgM fragments.

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The immunoglobulin molecules and fragments thereof, may be of animal, for example mammalian, origin and may be for example of murine, rat, or human origin. They may be of polyclonal, but are preferably of monoclonal origin. They
5 may be prepared using well known immunological techniques. For example, any suitable host may be injected with a cytokine and the serum collected to yield, after appropriate purification and/or concentration (for example by affinity chromatography using immobilised cytokine as
10 the affinity medium), the desired polyclonal antibody to the cytokine. Alternatively, splenocytes or lymphocytes may be recovered from the cytokine injected host and immortalised using for example the method of Galfre, G.,
15 et al (1977), Nature 266, 550, the resulting cells being segregated to obtain a single genetic line producing monoclonal antibodies to the antigen in accordance with conventional practice. Antibody fragments may be produced using conventional techniques, for example by enzymatic digestion of whole antibodies e.g. with pepsin
20 (Parham, J. Immunol., 131, 2895 (1983)) or papain (Lamoyi and Nisonoff, J. Immunol. Meth., 56, 235 (1983)).

Particularly useful immunoglobulins for use according to the invention include recombinant immunoglobulins and fragments thereof.

25 Especially useful recombinant immunoglobulins include:
(1) those having antigen binding domains at least part of which is derived from a different immunoglobulin, for example those in which the hypervariable or complementarity determining regions (CDRs) of one
30 immunoglobulin have been grafted into the variable domain framework of a second, different immunoglobulin (as described in European Patent Specification No. 239400);

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- 5 (2) recombinant immunoglobulins or fragments wherein non-variable domain sequences have been substituted by non-variable domain sequences from other, different immunoglobulins (as described in European Patent Specifications Nos. 120694, 125020, 171496, 173494 and 194276); or
- 10 (3) recombinant immunoglobulins or fragments possessing substantially the structure of a natural immunoglobulin but wherein the hinge region has a different number of cysteine residues from that found in the natural immunoglobulin, or wherein one or more cysteine residues in a surface pocket of the recombinant immunoglobulin or fragment is in the place of another amino acid residue present in the
- 15 natural immunoglobulin (as described in published International Patent Applications Nos. WO89/01974 and WO89/01782 respectively).

20 Most especially the recombinant immunoglobulins comprise humanised CDR-grafted or humanised chimeric immunoglobulins and fragments thereof.

Recombinant immunoglobulins may comprise whole immunoglobulin molecules or immunoglobulin molecule fragments, including those discussed above, as well as single chain antibodies, e.g. single chain Fvs.

25 Antigen binding domains of the multivalent immunoglobulin may be linked by means of a covalent cross-link. The cross-link may join antigen binding domains directly (for example as described in PCT/GB 90/00935 for multivalent Fvs) or, where an antigen binding domain is present as

30 part of a larger immunoglobulin fragment or a whole immunoglobulin molecule then the cross-link may be remote from the antigen binding domain.

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Immunoglobulin molecules or fragments thereof which include antigen binding domains may be linked by means of a disulphide bridge between cysteine residues or, alternatively, by means of a cross-linker molecule. The cross-linker may, generally, be one which is reactive with side chains of amino acids in the immunoglobulin molecules or fragments to be linked. Such amino acids preferably do not participate directly in antigen binding. Suitable amino acids for cross-linking include those with a side chain containing an amino, sulphhydryl, carboxyl, phenolic or other aromatic or heteroaromatic functional group through which a cross-linker may be attached. Suitable amino acids include lysine, cysteine, glutamic and aspartic acids, and tyrosine. Alternatively, the components of the multivalent immunoglobulin may be linked through carbohydrate residues of the components and, in particular, through oxidised carbohydrate residues. Suitable molecules for cross-linking immunoglobulins and other proteins are well known to those of skill in the art. A list of suitable molecules may be found, for example, in the 1989 Handbook and General Catalog of Pierce Chemical Company. One example of a way in which immunoglobulin molecules or fragments may be cross-linked is by reacting one component to be linked with 2-iminothiolane while activating the other with maleimide for example by reacting it with a maleimido-succinimide ester.

As an alternative to covalent linking antigen binding domains may be linked by non-covalent interactions for example, the link may be provided by an antibody molecule specific for sites on the immunoglobulin fragments or molecules to be cross-linked, these sites being ones which are not involved in antigen binding. For example where whole immunoglobulins are to be cross-linked this may be

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effected by means of an anti-Fc antibody directed to the constant region of the immunoglobulins to be linked.

5 An alternative way by which non-covalent cross-linking may be achieved is by biotin-avidin cross-linking in which immunoglobulin molecules or fragments derivatised with biotin are caused to aggregate through interaction with avidin.

10 Whatever the form of cross-linking used it is desirable that the antigen-binding domains in the multivalent immunoglobulin should be in the correct orientation for antigen binding.

15 As already mentioned preferred multivalent immunoglobulins of the present invention include linked IgG molecules and/or IgG fragments. However, the multivalent immunoglobulin may also be based on IgM molecules or fragments. For example, the multivalent immunoglobulin might be a fragment of a natural IgM for example obtained by partial enzymatic digestion. Alternatively, an artificial IgM might be produced by replacing the natural
20 cross-links of the IgM with others, possibly at a different location, and such cross-links could be used to link whole IgM molecules or fragments. A further possibility is that intact IgM pentamers could be linked to form decamers and etc.

25 According to a second aspect of the present invention there is provided a multivalent immunoglobulin of the first aspect of the invention for use in therapy.

30 The therapies in which the multivalent immunoglobulin of the present invention may be utilised include any in which it is desirable to reduce the level of or inhibit the

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activity of cytokine present in the human or animal body. The cytokine or lymphokine may be one which is in circulation in the patient or which is present in an undesirably high level localised at a particular site in the body. Elevated levels of $\text{TNF-}\alpha$, for example, are implicated in immunoregulatory and inflammatory disorders and in septic, or endotoxic, and cardiovascular shock. A multivalent immunoglobulin with antigen binding domains specific for $\text{TNF-}\alpha$ may be utilised in therapy of conditions which include: septic or endotoxic shock, cachexia, adult respiratory distress syndrome, AIDS, allergies, psoriasis, T.B., inflammatory bone disorders, blood coagulation disorders, burns, rejection episodes following organ or tissue transplant and autoimmune diseases e.g. organ specific diseases such as thyroiditis or non-specific organ diseases such as rheumatoid and osteo-arthritis.

Additionally, the multivalent immunoglobulin may be used to ameliorate side effects associated with TNF generation during neoplastic therapy and also to eliminate or ameliorate shock related symptoms associated with the treatment or prevention of graft rejection by use of an antilymphocyte antibody.

Similarly the multivalent immunoglobulins of the invention having specificity for other cytokines, such as interferon γ , lymphotoxin, IL-1, IL-2, IL-5 and IL-6 may be used to treat conditions associated with elevated levels of the cytokine in question.

According to a third aspect of the invention there is provided the use of a multivalent immunoglobulin according to the first aspect of the invention in the manufacture of a medicament for the treatment of a condition involving

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undesirably high levels of a cytokine for which the multivalent immunoglobulin is specific.

Preferably, the cytokine is interferon- γ (IFN- γ), interleukin-1 (IL-1), interleukin-2 (IL-2) interleukin-6 (IL-6), tumour necrosis factor- α (TNF- α), or tumour necrosis factor- β (TNF- β).

The medicament prepared according to the third aspect of the invention may be for administration in any appropriate form and amount according to the therapy in which it is employed. It may be for prophylactic use, for example where circumstances are such that an elevation in the level of a particular cytokine might be expected, or, alternatively, the medicament may be for use in reducing the level of the cytokine after it has reached an undesirably high level or as the level is rising.

According to a further aspect of the invention there is provided a pharmaceutical composition comprising a multivalent immunoglobulin according to the first aspect of the invention in combination with a pharmaceutically acceptable diluent, excipient or carrier.

The composition may comprise other active ingredients. The composition may take any suitable form for administration, and, in particular, will be in a form suitable for parenteral administration e.g. by injection or infusion, for example by bolus injection or continuous infusion. Where the composition is for injection or infusion it may take the form of a suspension, solution or emulsion of the multivalent immunoglobulin in an oily or aqueous vehicle and it may contain formulatory agents such as suspending, stabilising and/or dispersing agents.

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Alternatively, the composition may be in dry form, for reconstitution before use with an appropriate sterile liquid.

5 The dose at which the multivalent immunoglobulin will be administered will depend on the nature of the condition to be treated, the degree to which the cytokine to be neutralised is, or is expected to be, raised above a desirable level, on whether the multivalent immunoglobulin is being used prophylactically or to treat an existing
10 condition, and on the nature of the particular multivalent immunoglobulin selected for the therapy. Dose will also be selected according to the age and condition of the patient.

15 Thus, for example, where the multivalent immunoglobulin is a conjugate of intact IgG molecules having specificity for TNF- α , the IgG molecules being cross-linked with an anti-Fc antibody, and where the multivalent immunoglobulin is employed in the prophylaxis or treatment of TNF- α related conditions such as shock or inflammation suitable
20 doses are in the range 0.001 to 10 mg/kg/day. The dose may be continued for as long as is necessary to alleviate the condition associated with the undesirably high level of antigen.

25 In a still further aspect of the invention there is provided a method of therapeutic treatment of a human or animal subject suffering from a disorder associated with an undesirably high level of a cytokine, the method comprising administering to the subject an effective amount of a multivalent immunoglobulin according to the
30 first aspect of the invention.

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In another aspect of the invention there is provided a process for the production of a multivalent immunoglobulin of the first aspect of the invention said method comprising linking the immunoglobulin molecules or
5 fragments thereof by means of a suitable linker.

Suitable linkers have already been mentioned above. It will therefore be understood that linking may be effected by reacting immunoglobulin molecules or fragments with a covalent cross-linker or by bringing them into contact
10 with an antibody or other aggregating agent to effect non-covalent cross-linking.

Also in the case of recombinant immunoglobulins, processes for the production of the multivalent immunoglobulins will involve expression of immunoglobulin molecules or
15 fragments in appropriate host cells transformed with suitable DNA sequences.

BRIEF DESCRIPTION OF THE FIGURES

The invention is described below by way of example with reference to the following figures of which:

- 20 Figure 1 is a plot of residual bioactive TNF- α activity after antibody treatment against initial concentration of anti-TNF antibody for 4 different antibodies designated 129/16, 101/4, CB6 and 27/26;
- 25 Figure 2 is a sketch showing the manner in which anti-TNF antibodies may be linked by means of an anti-Fc (it will be appreciated that the array, illustrated in two dimensions is, in fact, three dimensional);

- 5 **Figures 3-5** show plots of residual bioactive TNF- α after antibody treatment against initial concentration of anti-TNF for three anti-TNF antibodies (101/4, HTNF1 and CB6) which were cross-linked with a polyclonal anti-Fc;
- Figure 6** shows a plot of TNF- α activity remaining after antibody treatment against concentration of biotinylated CB6 antibody cross-linked with avidin;
- 10 **Figure 7** compares the neutralising efficiency of a biotinylated anti-lymphotoxin antibody (hLT12 Biotin) to the same antibody after avidin cross-linking (hLT12 Biotin:Avidin 1:1);
- 15 **Figure 8** shows a plot of TNF- α activity remaining after antibody treatment against concentration of CB6 antibody which has been covalently cross-linked;
- Figure 9** is a plasmid diagram of plasmid pE2056 containing the human C μ coding sequence, and
- 20 **Figure 10** shows plots of residual bioactive TNF- α after antibody treatment against initial concentration of anti-TNF for IgG and IgM versions of the antibody 101/4; numerals 2-5 refer to four separate samples of 101/4 IgM.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTIONExample 1Comparative Example IgG and IgM

Antibodies 129/16 and 101/4 are murine monoclonal
5 antibodies of immunoglobulin class IgG raised against
recombinant human TNF- α and obtained from the National
Institute for Biological Standards and Controls (NIBSC).
CB6 and HTNF1 are also murine monoclonal IgG to
recombinant human TNF- α and were produced at Celltech.
10 Antibody 27/26 is a murine monoclonal IgM against TNF- α
and was obtained from NIBSC.

Comparison of Neutralising Activity of IgGs and IgM

The neutralising activity of various antibodies was
compared using an L929 bioassay as described by Ruff and
15 Gifford, Lymphokines 2: 235 (1981). Briefly, it was
carried out as follows.

L929 cells were prepared for use in the following way.

The supernatant medium was removed from L929 cells which
were growing as an adherent monolayer in RPMI 1640 medium
20 (Gibco) containing 2mM glutamine 10% FCS, and 100 μ g/ml
gentamicin (RP10). The monolayer was washed in
Dulbecco's A phosphate buffered saline (PBS) (Gibco).
Trypsin EDTA (Gibco) was added for 10 min to remove cells
from the plastic and the cell suspension was then added to
25 RP10 medium at a final density of 3×10^5 viable cells/ml
as assessed by Trypan Blue (Gibco) dye exclusion. 100 μ l
of this cell suspension was then added to each well of
flat-bottomed 96-well microtitre trays (Falcon Microtest)
and incubated over-night at 37°C.

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Dilutions of the antibodies to be tested were prepared in assay medium (RPMI 1640 containing glutamine, gentamicin and 2% FCS 1% actinomycin D). Dilutions of human recombinant TNF- α (NIBSC standard) were also made up in assay medium then added to all dilutions.

The L929 assay was subsequently carried out as follows:

Microtitre plates containing the L929 cells were flicked to remove the RPMI 1640 medium. 100 μ l of the antibody and TNF dilutions were dispensed to the appropriate wells of the microtitre plates. Control wells containing assay medium and antibodies at the highest concentration were added. The plates were then incubated overnight at 37°C.

After the overnight incubation, the L929 plates were flicked to remove the medium and were washed once carefully in PBS. Methanol (AnalaR) was then added to all wells and left for 30-60 seconds. The methanol was then flicked off, a solution of crystal violet (1% w/v in water) was added to all the wells and incubated for 5 mins. This was then washed off with tap water and the stain then dissolved in the well in 100 μ l of 30% glacial acetic acid (AnalaR). Absorbance was measured at 570nm and the absorbance at 410nm was used as a reference measurement to correct for light scatter.

Live L929 cells take up crystal violet while those killed by TNF do not. In experiments where the amount of TNF added to the wells is kept constant and the antibody concentration is varied the absorbance of the extracted stain may therefore be used as a measure of the TNF available in solution for killing L929 cells.

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The results of the experiment in which antibodies 129/16, 101/4, CB6 and 27/26 were compared are shown in Figure 1. In this case the concentration of TNF in each well was 400pg/ml which is sufficient to effect almost total cell killing; the concentration of the various anti-TNF antibodies varied from well to well.

It is apparent from the results shown that significantly greater amounts of the IgG antibodies than of the IgM antibodies were required to neutralise the same amount of TNF- α . Thus for example where the starting concentration of TNF- α was 400pg/ml the ID₅₀ (the concentration of antibody that gave 50% inhibition) was 3.4ng/ml for the IgM antibody 27/26, whereas the ID₅₀s for the IgG antibodies were 55ng/ml for 129/16, 42ng/ml for CB6 and 11ng/ml for 101/4.

Thus, it is clear that the IgM antibody has a significantly higher neutralising activity than corresponding IgGs.

In order to establish whether the increased neutralising activity of the IgM was a result of the cross-linking of the immunoglobulin units various IgGs were cross-linked and the effect on their neutralising activities investigated.

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Example 2Cross-linking Antibodies Using Anti-Fc

5 Selected IgGs were cross-linked using a polyclonal anti-Fc antibody (Jacksons Labs) specific for the constant region of the murine IgG molecule. The anti-Fc antibody has the effect of cross-linking the IgG's by their Fc regions leaving their binding domains available for antigen binding. The manner of binding is represented in Figure 2 in which IgG anti-TNF molecules 1 are arranged in an array linked at the N terminal ends 3 of their constant regions 5 by anti-Fc antibody molecules 7. TNF molecules 9 are shown bound at the antibody binding sites at the C terminal ends 11 of the anti-TNF molecules 1. From 2 up to around 2000 anti-TNF molecules may be cross-linked together in this way.

The following procedure was used to effect the cross-linking.

20 Separate solutions of each antibody to be tested together with goat [F(ab')₂] anti-mouse IgG-Fc in L929 assay medium were made up to a concentration of 2µg/ml antibody and 20µg/ml anti-Fc. Up to nine, three-fold dilutions of the antibody-anti-Fc mixtures were prepared and the dilutions incubated at 37°C for 1 hour.

25 The neutralising activity of the cross-linked antibodies was assessed using an L929 bioassay as described above. 100µl of each of the antibody - anti-Fc mixture and of TNF being dispensed into the appropriate wells of a microtitre tray. The concentration of TNF in each well was 400pg/ml as before.

30 The effect of the cross-linking is demonstrated in Figures 3-5 in which TNF remaining available for cell killing is

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plotted against antibody dilution for each of the IgG antibodies 101/4, HTNF1 and CB6. In each case measurements were made for the antibody alone or with added anti-Fc. Anti-Fc alone was shown to have no effect.

5 Figure 3, for example, shows the effect of adding anti-Fc antibody to the anti-TNF antibody 101/4. It is apparent from this plot that for a given antibody dilution more TNF is neutralised in the presence than in the absence of cross-linking by anti-Fc.

10 Similar effects are demonstrated in Figures 4 and 5 for the antibodies HTNF1 and CB6 respectively. It is noteworthy that the poorer the neutralising activity of the antibody in the absence of anti-Fc the greater the improvement in neutralising activity which can be brought
15 about by the addition of the anti-Fc antibody.

Table 1 below summarises the effect of polyclonal anti-Fc on TNF neutralisation by each of the antibodies 101/44, HTNF1 and CB6. In each case neutralisation was improved
20 to an efficacy that is close to the theoretical maximum, i.e. a molar excess of antibody to antigen of 0.5-1.

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Table I

Summary Table showing effect of a polyclonal anti-Fc on efficacy of anti-TNF antibodies in TNF neutralisation

	<u>Anti-TNF</u> <u>Alone</u>	<u>Anti-TNF</u> <u>+ Anti-Fc</u>	<u>Fold</u> <u>Improvement</u>
<u>Molar excess of antibody required for</u> <u>50% inhibition of TNF</u>			
101/4	1.3	0.3	4.3
HTNF	7	1	7
CB6	60	0.4	150

Example 3Cross-Linking Antibodies Using Biotin-Avidin

[a] Anti-TNF antibody CB6 was biotinylated according to conventional procedure as described, for example, in Practical Immunology, L. Hudson and F.C. Hay, 3rd Edn, 1989, Blackwell Scientific Publications.

Biotin labelled antibody was mixed with streptavidin (Jackson Labs) at molar ratios of 10:1, 1:1 and 1:10, and then incubated at 37°C for 1hr to form complexes. Three fold serial dilutions of the biotin labelled antibody and of the complexes were prepared in L929 assay medium and the L929 assay carried out as described above.

The results are shown in Figure 6 from which it is apparent that where biotinylated CB6 and avidin were complexed in a 1:1 ratio the neutralising activity of the antibody was significantly increased relative to uncomplexed biotinylated antibody or to complexes formed with 10:1 or 1:10 ratios of biotinylated antibody to avidin.

[b] The experiment in Part [a] was repeated using a biotinylated anti-lymphotoxin antibody (hLT12) cross-linked with avidin at a molar ration of 1:1. The results are shown in Figure 7 from which it is apparent that the neutralising activity of the cross-linked antibody was significantly increased relative to uncomplexed biotinylated antibody.

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Example 4Chemical Cross-Linking of Antibodies

Chemically cross-linked CB6 was prepared as follows:

5 1.65mg of CB6 (5mg/ml in PBS) was incubated with 3.2mg of 2-iminothiolane in 1ml of ethanol with gentle agitation for 30 minutes at room temperature.

4.75mg of CB6 (5mg/ml in PBS) was incubated with 0.195mg maleimidohexanoyl succinimide ester, with gentle agitation for 45 minutes at room temperature.

10 In each case excess reactants were removed by chromatography on a PD10 column.

The iminothiolane and maleimido derivatives of CB6 were then reacted together at a ratio of 1:1 by incubation together at room temperature for two hours.

15 Ethyl maleimide in DMSO was then added to each incubation to give a final concentration of 0.01mg/ml.

20 The reaction products were subjected to HPLC, the absorbance of the fractions at 280nm being used to locate protein containing fractions. Selected column fractions were pooled and employed in L929 assays, as described previously.

25 The results of the cross-linking experiments are shown in Figure 8. Fraction #6 corresponds to the monomer and Fractions #5 to #1 contain complexed antibody of increasing molecular weight. [Fraction #7 is smaller than the monomer and probably represents an antigen binding degradation product.] It is apparent that, in general, chemical cross-linking of the antibody molecules results in an enhancement of the neutralising activity of the antibody.

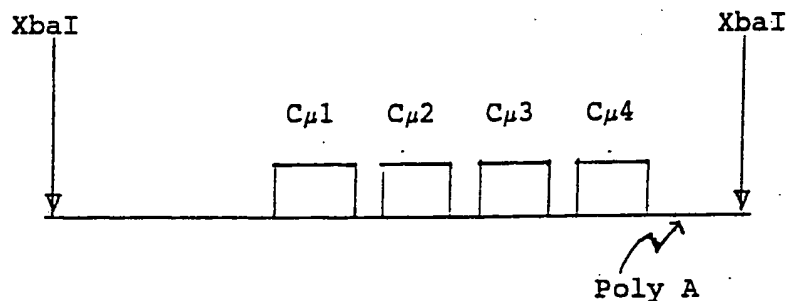
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Example 5Preparation of recombinant chimeric IgM

5 In a further experiment recombinant chimeric IgM anti-TNF antibodies were prepared and tested for their ability to neutralise TNF in the L929 bioassay.

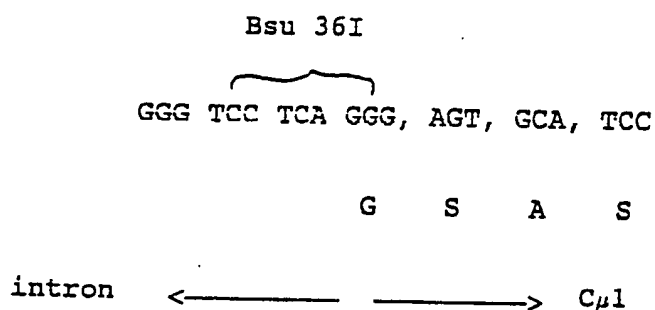
DNA coding for the light and heavy chain variable domains of the murine IgG anti-TNF antibody 101.4 were ligated with DNA coding for human K light chain constant region and human μ heavy chain constant region ($C\mu$) respectively.

10 Construction of IgM versions of 101.4 Heavy Chain
Human $C\mu$ DNA was obtained as a XbaI fragment (Ward, C.J. et al (1989) International Immunology 1, No. 3, 296-309) in which the coding sequences are arranged as shown in the following restriction map.

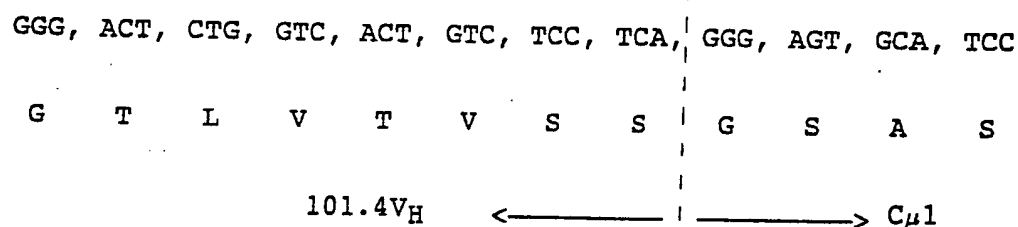


15 The XbaI fragment was cloned into the Xba site of pEE7.HCMV [Stephens, P and Cockett, M.I. in Nucl. Acid. Res. 17, 7110 (1989)] to produce plasmid pE2056 as shown in Figure 9. pE2056 has a unique restriction site for
15 the enzyme Bsu 36I located at the start of $C\mu 1$. The
20 relevant sequence is shown below.

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Thus pE2056 was digested with HindIII and Bsu 36I and the larger fragment ligated with a HindIII-Bsu 36I fragment comprising the coding sequence of the heavy chain variable domain of 101.4 to produce the following sequence across the V-C μ 1 junction of the new genes.



This procedure generated four plasmids (which should be identical) which were isolated and designated pE2058-pE2061 inclusive. In Figure 10 samples 2-5 correspond to results obtained using plasmids pE2058-pE2061 respectively.

Similarly a chimeric 101.4 variable-human K constant light chain was prepared and inserted into a suitable expression plasmid (substantially as described in co-pending British Patent Application 9109645.3). COS cells were transfected with the gene coding for the chimeric light chain together with pE2058, pE2059, pE2060 and pE2061 each in turn. COS cell supernatants were assayed in the L929

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5 bioassay and the ability of the resultant recombinant chimeric "IgM" immunoglobulins to neutralise TNF compared with that of murine 101.4. The results obtained are given graphically in Figure 10 indicating that lower amounts of the recombinant chimeric "IgM" product than the murine IgG product were required for neutralisation of TNF.

10 The foregoing examples are given to illustrate the invention. It will be appreciated that other monoclonal antibodies to TNF and antibodies to other cytokines in general may be used for preparation of multivalent immunoglobulins according to the invention by alternative methods as well as by loosely following the general procedure set out above.

CLAIMS

1. A multivalent immunoglobulin comprising at least three linked antigen binding domains each of said domains being specific for a complementary site on a cytokine.
- 5 2. A multivalent immunoglobulin according to Claim 1 in which the combined interactions between the antigen binding domains of the immunoglobulin and the complementary cytokine sites are neutralising interactions.
- 10 3. A multivalent immunoglobulin according to Claim 1 or 2 which is specific for $\text{TNF-}\alpha$, $\text{TNF-}\beta$, an interleukin, an interferon, or a colony stimulating factor.
- 15 4. A multivalent immunoglobulin according to any one of the preceding claims which has specificity for a cytokine which is naturally present as a multimer.
5. A multivalent immunoglobulin according to any one of the preceding claims comprising 4 to 20 antigen binding domains.
- 20 6. A multivalent immunoglobulin according to any one of the preceding claims in which the antigen binding domains are all of class IgG.
- 25 7. A multivalent immunoglobulin according to any one of Claims 1 - 5 in which the antigen binding domains are all of class IgM and differs from a native IgM molecule.
8. A multivalent immunoglobulin according to any one of the preceding claims comprising recombinant immunoglobulins and fragments thereof.

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9. A multivalent immunoglobulin according to any one of the preceding claims in which antigen binding domains are linked by covalent cross-links.
- 5 10. A multivalent immunoglobulin according to any one of Claims 1 - 8, in which antigen binding domains are linked by non-covalent interactions.
11. A multivalent immunoglobulin according to Claim 1 for use in therapy.
- 10 12. The use of a multivalent immunoglobulin according to Claim 1 in the manufacture of a medicament for the treatment of a condition involving undesirably high levels of a cytokine for which the multivalent immunoglobulin is specific.
- 15 13. A pharmaceutical composition comprising a multivalent immunoglobulin according to Claim 1, in combination with a pharmaceutically acceptable diluent, excipient or carrier.
- 20 14. A method of therapeutic treatment of a human or animal subject suffering from a disorder associated with an undesirably high level of a cytokine, comprising administering to the subject an effective amount of a multivalent immunoglobulin according to Claim 1.
- 25 15. A process for the production of a multivalent immunoglobulin according to Claim 1 comprising linking the immunoglobulin molecules or fragments thereof together by means of a suitable linker.

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Neutralization of TNF by various anti-TNF antibodies

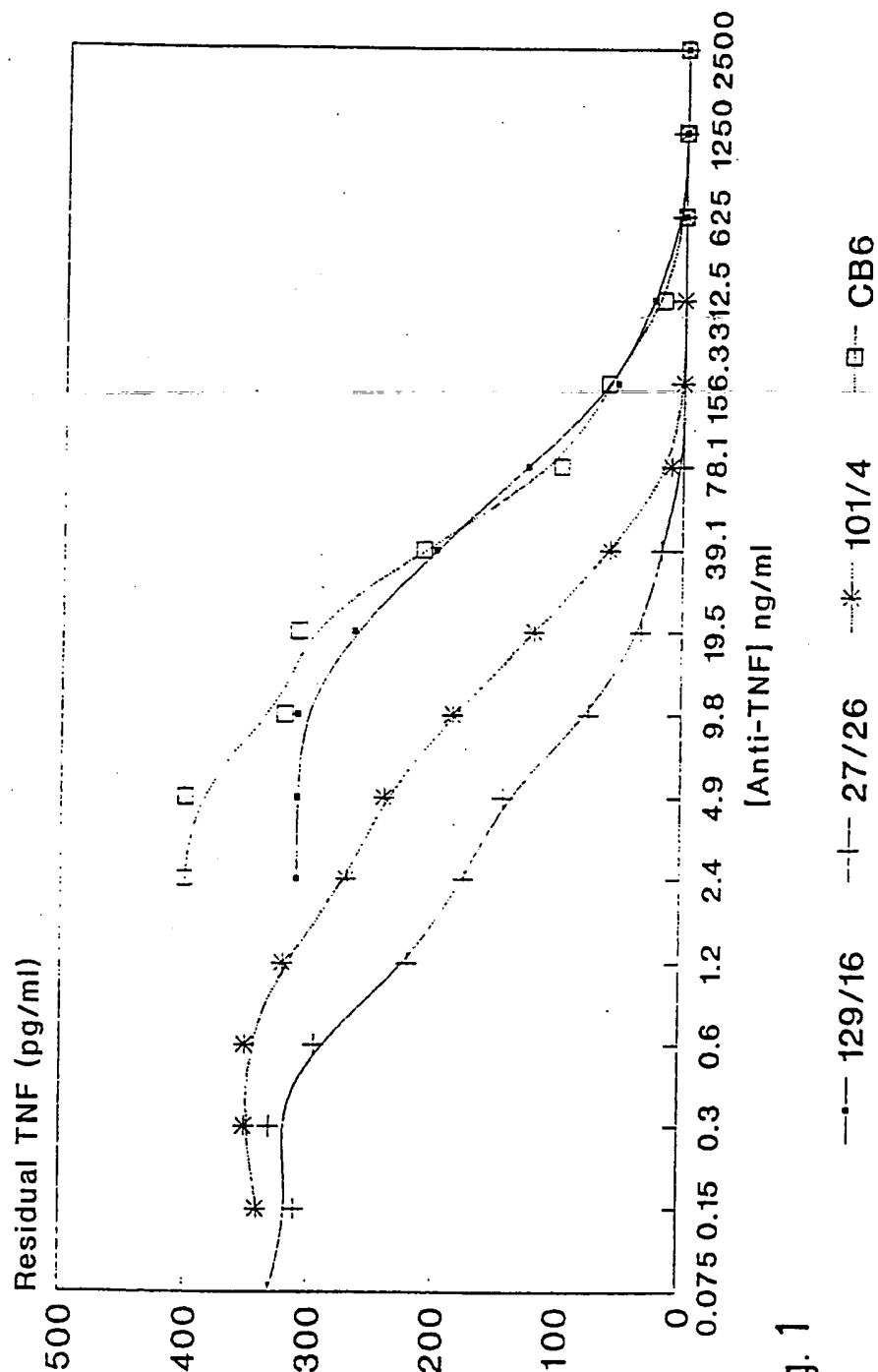


Fig. 1

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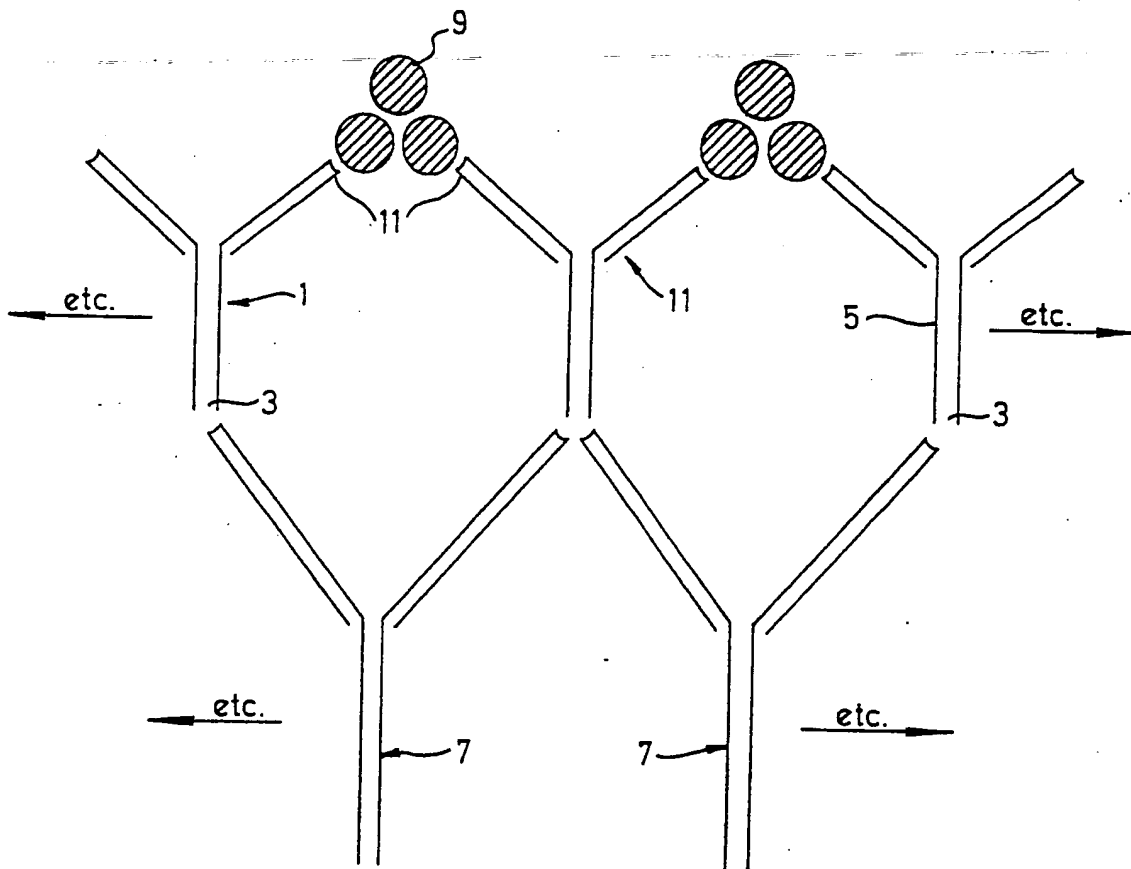


Fig. 2

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Effect of cross-linking 101/4
in the TNF neutralisation assay

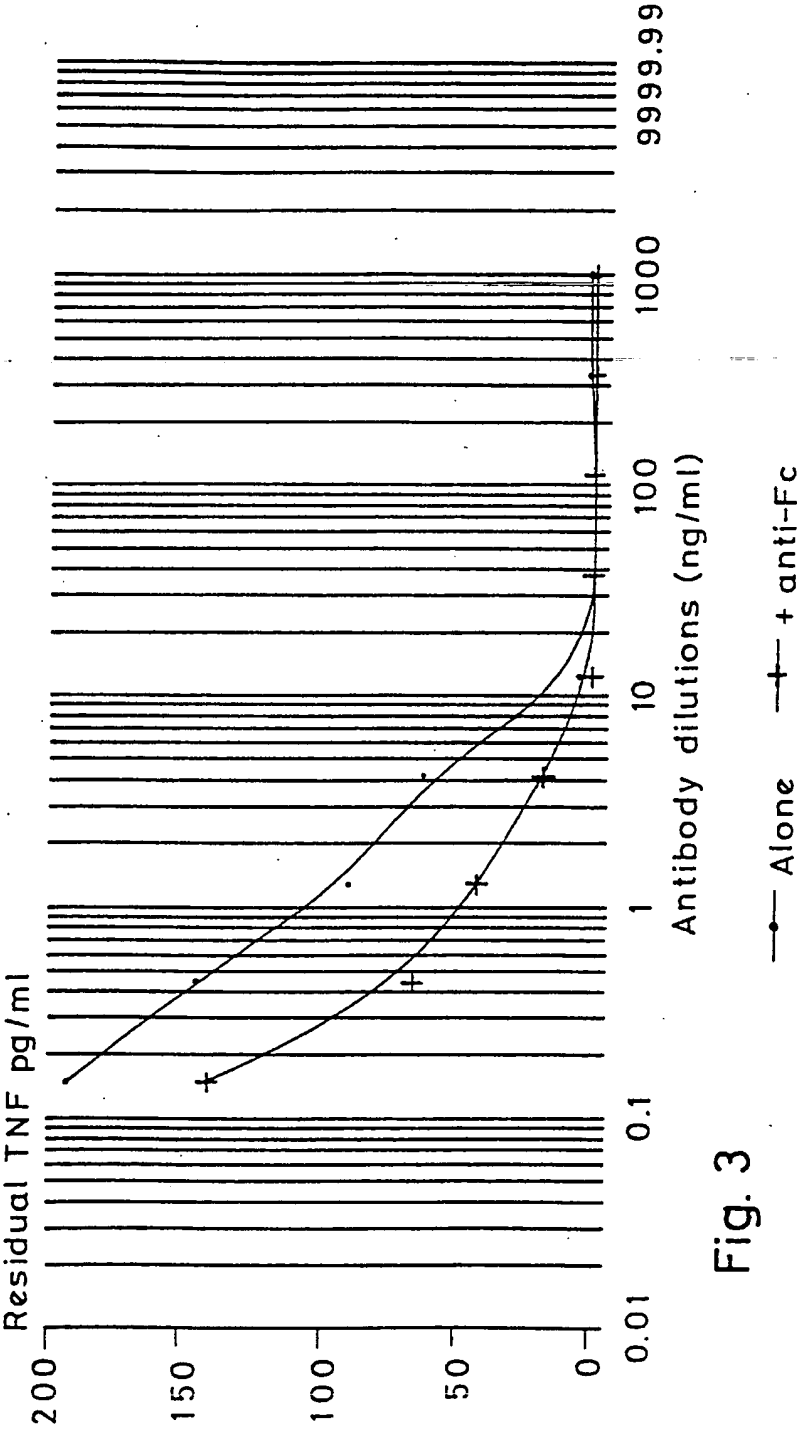


Fig. 3

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Effect of cross-linking HTNF1
in the TNF neutralisation assay

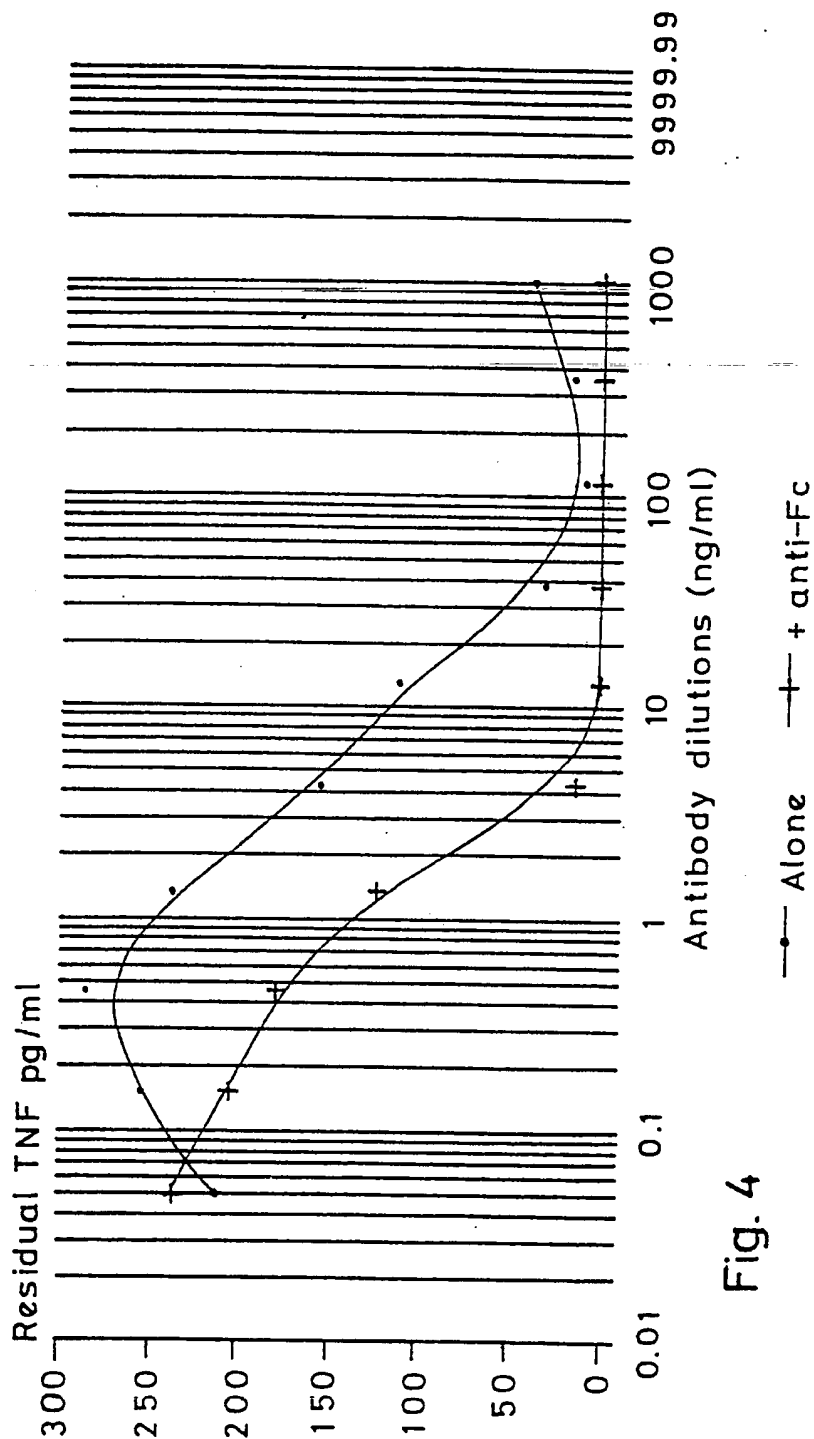


Fig. 4

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Effect of cross-linking CB6
in the TNF neutralisation assay

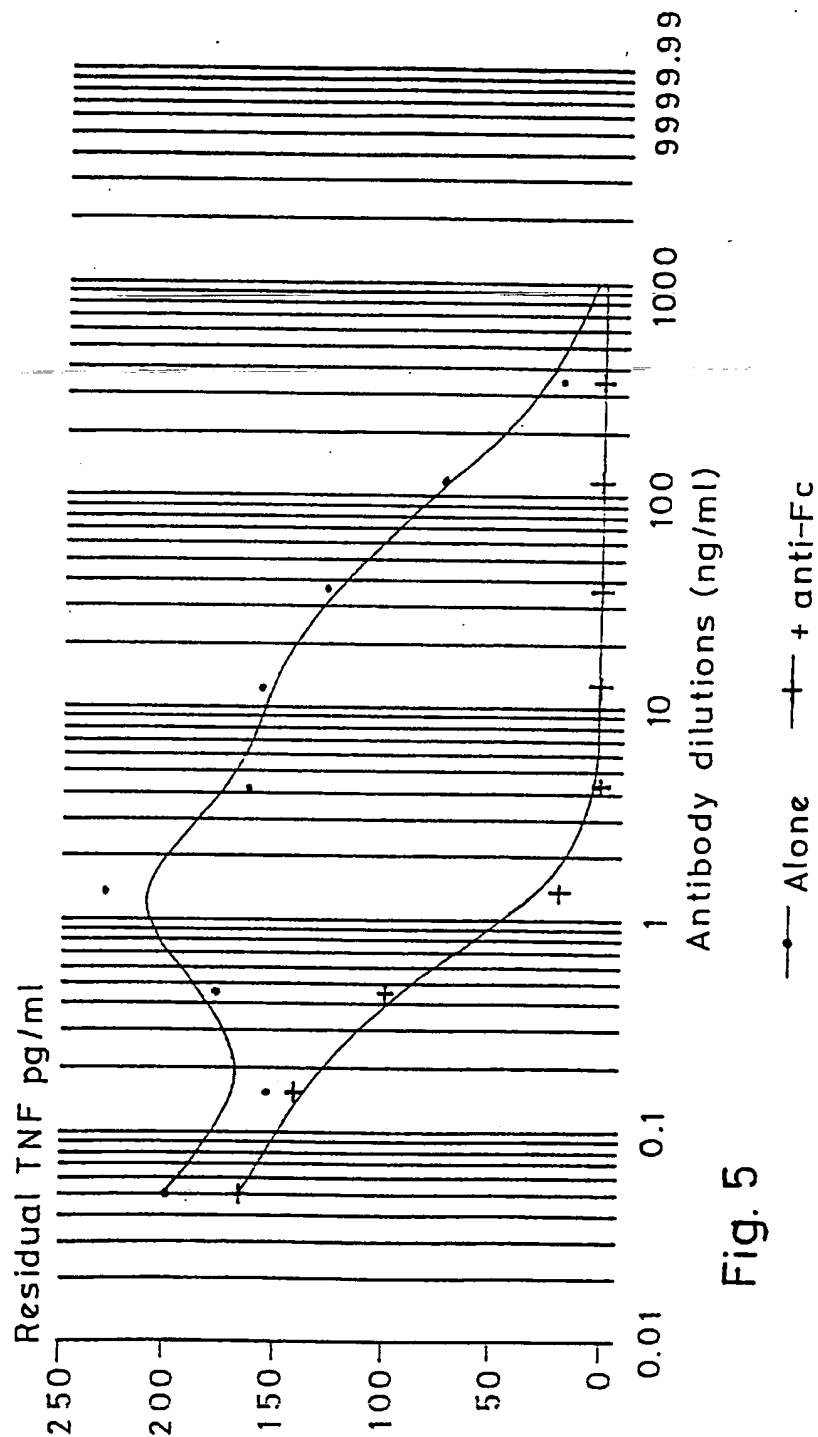


Fig. 5

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Avidin Crosslinking of Biotinylated-CB6

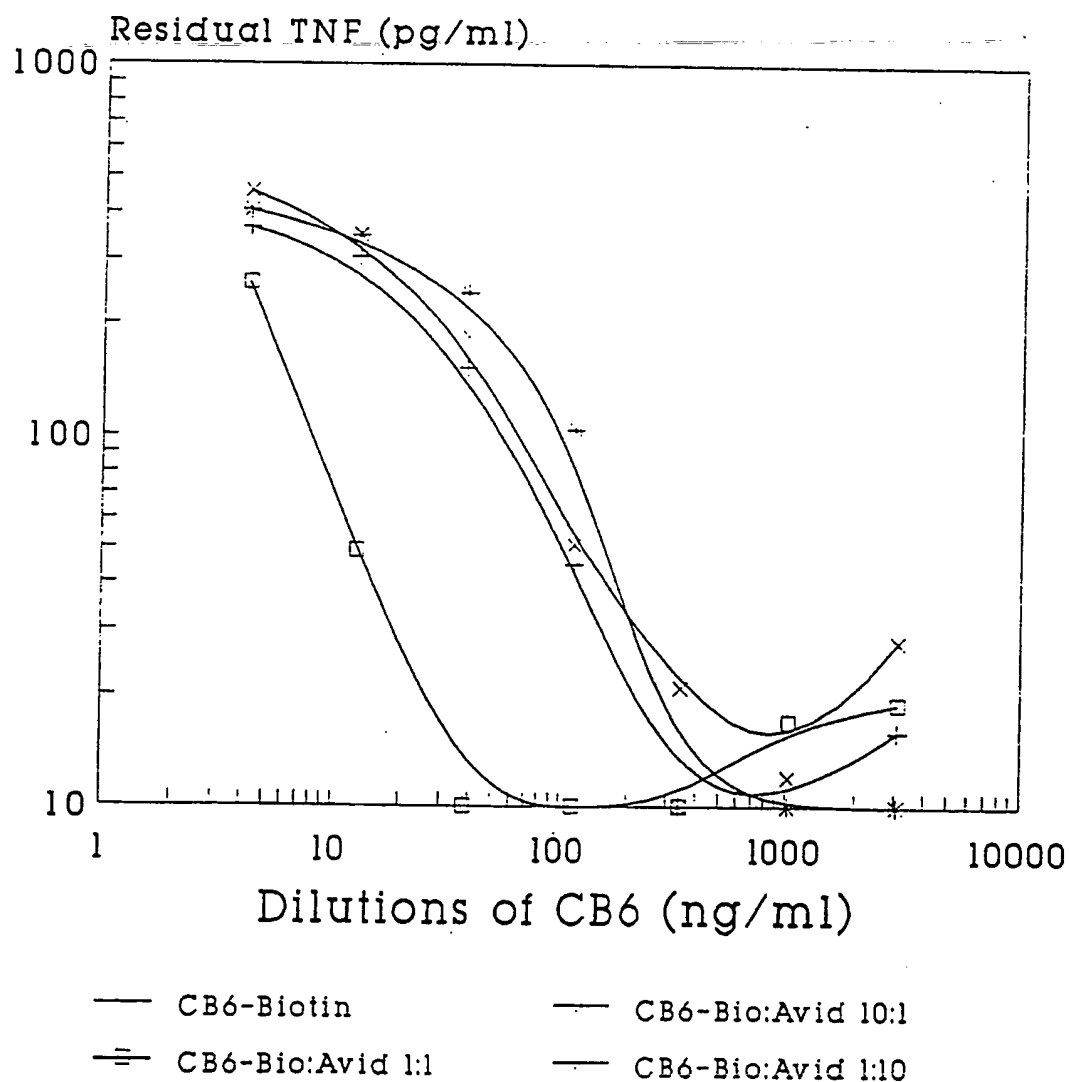


Fig. 6

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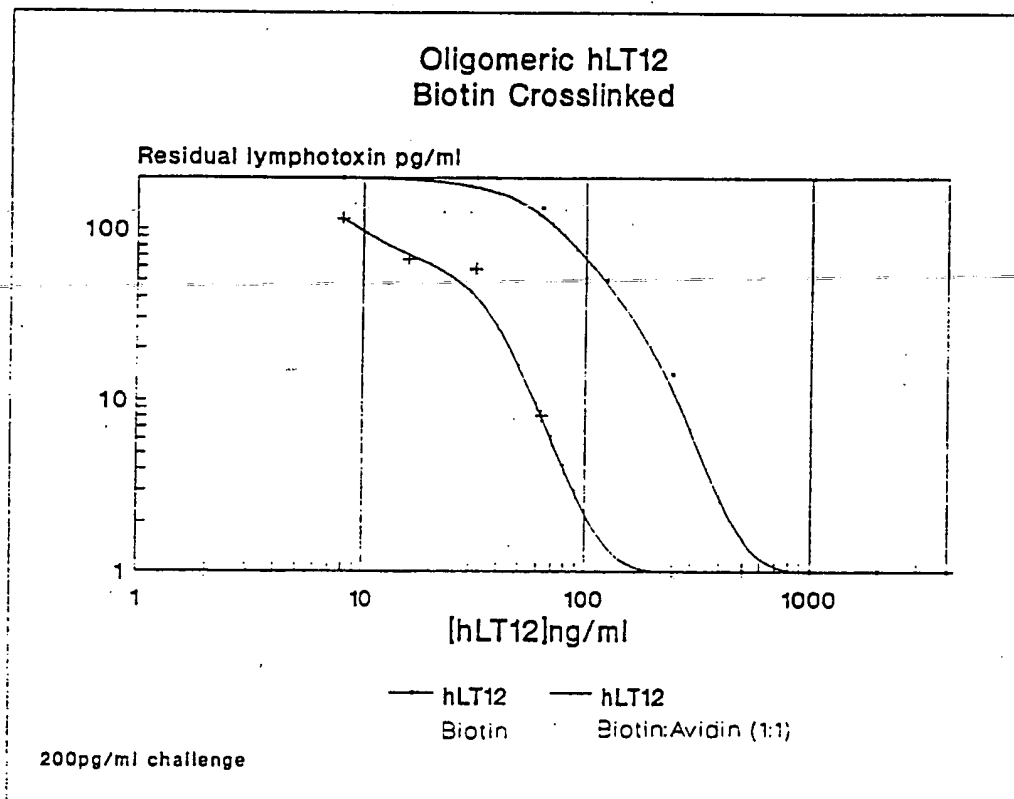
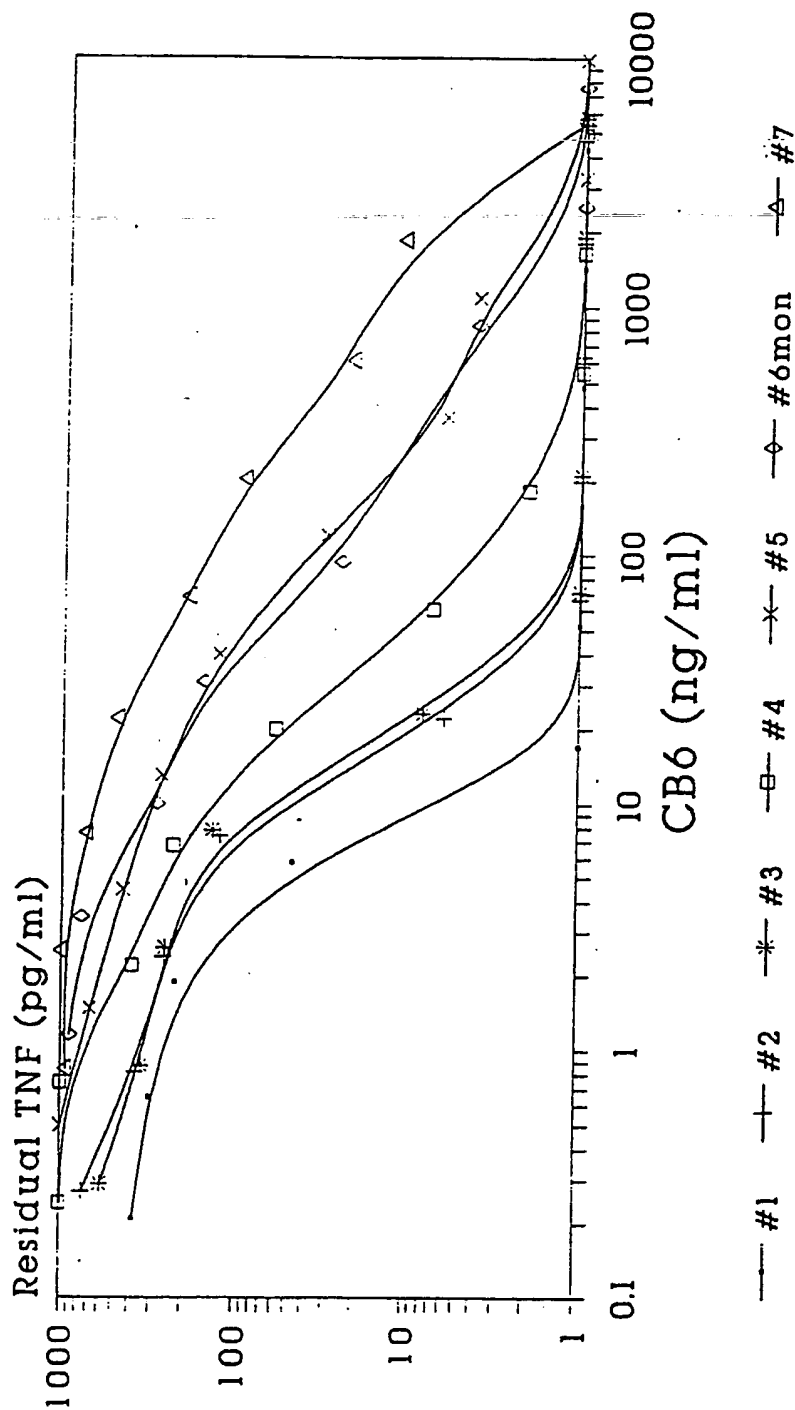


Fig. 7

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Covalently Crosslinked CB6 Thiol-Maleimide linkage

Fig. 8



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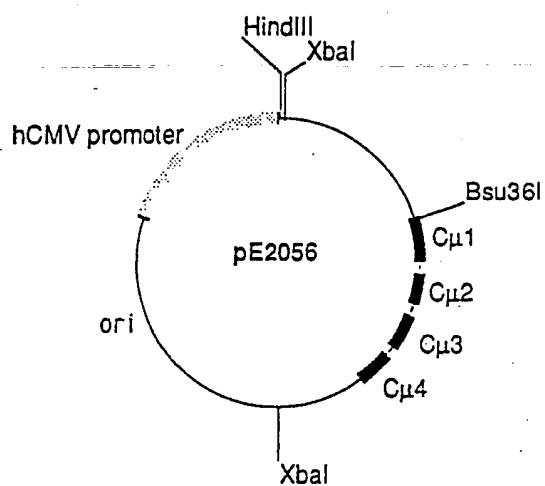
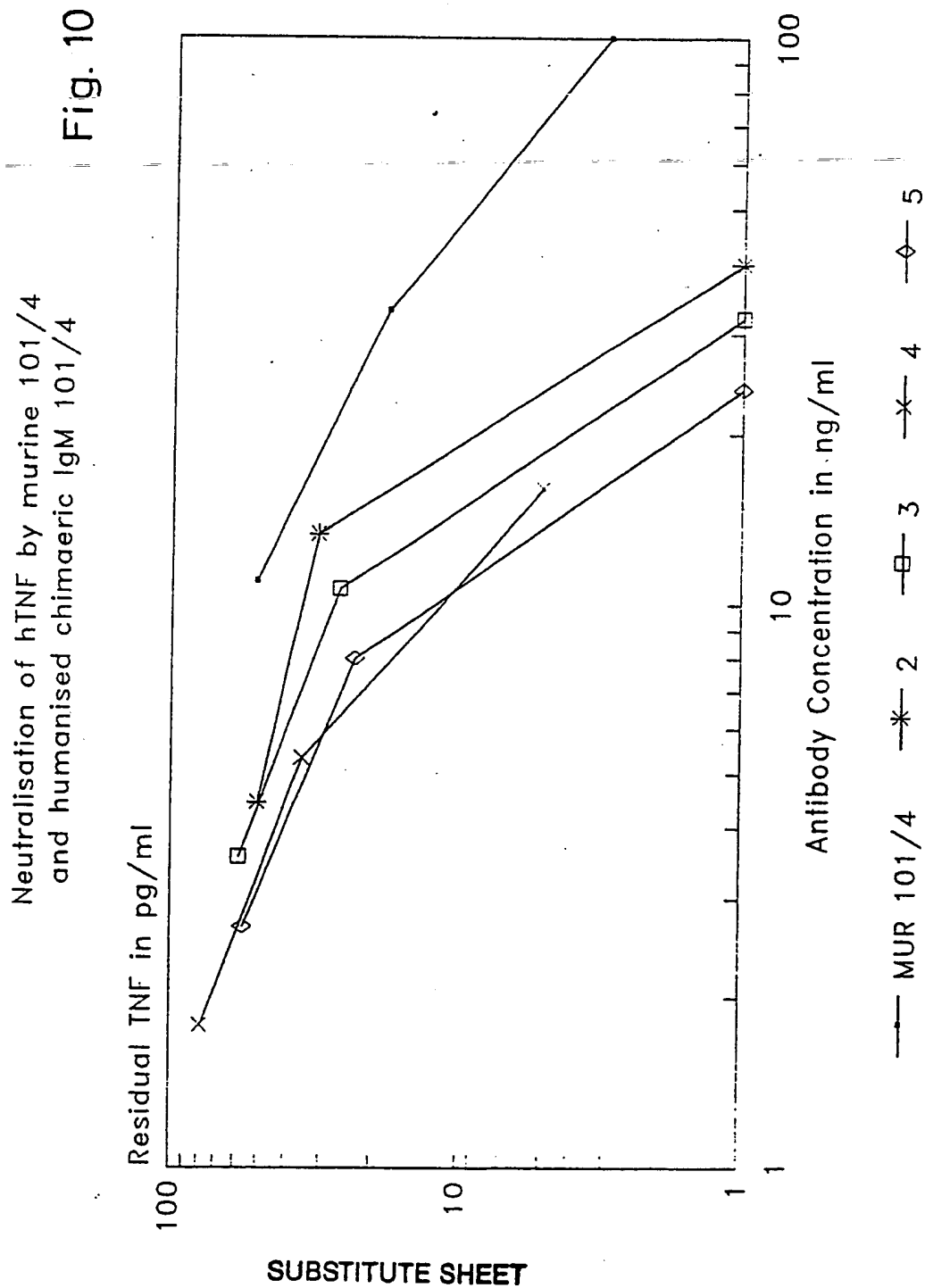


Fig. 9



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01216

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: A 61 K 39/395, C 12 P 21/08, C 07 K 15/28														
II. FIELDS SEARCHED <div style="text-align: center;">Minimum Documentation Searched⁷</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%;">Classification System</th> <th style="width: 80%;">Classification Symbols</th> </tr> <tr> <td style="height: 40px; vertical-align: bottom;">IPC5</td> <td style="vertical-align: bottom;">A 61 K; C 12 P; C 07 K</td> </tr> </table> <div style="text-align: center;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched⁸</div>			Classification System	Classification Symbols	IPC5	A 61 K; C 12 P; C 07 K								
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III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category¹⁰</th> <th style="width: 60%;">Citation of Document¹¹ with indication, where appropriate, of the relevant passages¹²</th> <th style="width: 30%;">Relevant to Claim No.¹³</th> </tr> </thead> <tbody> <tr> <td style="vertical-align: top;">P,A</td> <td>WO, A1, 9106305 (BRISTOL-MYERS SQUIBB COMPANY) 16 May 1991, see the whole document --</td> <td style="vertical-align: top;">1-13, 15</td> </tr> <tr> <td style="vertical-align: top;">Y</td> <td>WO, A3, 9007118 (BAJYANA-SONGA) 28 June 1990, see the whole document --</td> <td style="vertical-align: top;">1-13, 15</td> </tr> <tr> <td style="vertical-align: top;">Y</td> <td>Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 07356365, Boughton BJ et al: "The effect of anti-Rho(D) and non-specific immunoglobulins on monocyte Fc receptor function: the role of high molecular weight IgG polymers and IgG subclasses", & Clin Lab Haematol 1990, 12 (1) p17-23 --</td> <td style="vertical-align: top;">1-13, 15</td> </tr> </tbody> </table>			Category ¹⁰	Citation of Document ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	P,A	WO, A1, 9106305 (BRISTOL-MYERS SQUIBB COMPANY) 16 May 1991, see the whole document --	1-13, 15	Y	WO, A3, 9007118 (BAJYANA-SONGA) 28 June 1990, see the whole document --	1-13, 15	Y	Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 07356365, Boughton BJ et al: "The effect of anti-Rho(D) and non-specific immunoglobulins on monocyte Fc receptor function: the role of high molecular weight IgG polymers and IgG subclasses", & Clin Lab Haematol 1990, 12 (1) p17-23 --	1-13, 15
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<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search 25th November 1991 </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report 18. 12. 91 </td> </tr> <tr> <td style="width: 50%; padding: 5px;"> International Searching Authority EUROPEAN PATENT OFFICE </td> <td style="width: 50%; padding: 5px;"> Signature of Authorized Officer NURIA TORAL </td> </tr> </table>			Date of the Actual Completion of the International Search 25th November 1991	Date of Mailing of this International Search Report 18. 12. 91	International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer NURIA TORAL								
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP, A1, 0355067 (CEL TECH LIMITED) 21 February 1990, see the whole document --	1-13, 15
Y	WO, A1, 9006371 (COMMISSARIAT A L'ENERGIE ATOMIQUE) 14 June 1990, see the whole document --	1-13, 15
Y	EP, A1, 0347057 (SCHERING BIOTECH CORPORATION) 20 December 1989, see the whole document -----	1-13, 15

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers...14..., because they relate to subject matter not required to be searched by this Authority, namely:

Method for therapeutic treatment of a human or animal subject, -c.f. PCT rule 39.4

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 91/01216**

SA 50532

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 31/10/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9106305	16/05/91	AU-D- 7030391	31/05/91
WO-A3- 9007118	28/06/90	FR-A-B- 2640143	15/06/90
EP-A1- 0355067	21/02/90	AU-D- 4182289 WO-A- 90/01950	23/03/90 08/03/90
WO-A1- 9006371	14/06/90	CA-A- 2004935 EP-A- 0399024 FR-A- 2640146	08/06/90 28/11/90 15/06/90
EP-A1- 0347057	20/12/89	AU-D- 3834589 EP-A- 0452315 JP-T- 3502582 WO-A- 89/11864	05/01/90 23/10/91 13/06/91 14/12/89

For more details about this annex : see Official Journal of the European patent Office, No. 12/82